



## INTERNATIONAL APPLICATION PUBLISHED

## (51) International Patent Classification 6 :

C12P 37/04, 35/04

A1

(43) International Publication Date: 1 February 1996 (01.02.96)

(21) International Application Number: PCT/EP95/02876

(22) International Filing Date: 18 July 1995 (18.07.95)

(30) Priority Data:  
853/94 18 July 1994 (18.07.94) DK

(71) Applicant (for all designated States except US): GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): CLAUSEN, Kim [DK/DK]; Hovedgaden 12, DK-4340 Tøløse (DK). DEKKERS, Rocus, Marinus [NL/NL]; Strausslaan 5, NL-2992 PC Barendrecht (NL).

(74) Agents: VISSER-LUIRINK, Gesina et al.; Gist-Brocades N.V., Patents and Trademarks Dept., Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

## Published

With international search report.

(54) Title: PROCESS FOR PREPARATION OF  $\beta$ -LACTAMS AT CONSTANTLY HIGH CONCENTRATION OF REACTANTS

## (57) Abstract

$\beta$ -Lactam derivatives can advantageously be synthesized by an enzymatic reaction of the parent amino  $\beta$ -lactam with the corresponding acylating agent at constantly high concentrations of said reactants.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

PROCESS FOR PREPARATION OF  $\beta$ -LACTAMS AT CONSTANTLY HIGH  
CONCENTRATION OF REACTANTS

TECHNICAL FIELD

5 This invention relates to a an improved process for preparation of  $\beta$ -Lactam derivatives by enzymatic acylation of the parent amino  $\beta$ -lactam with an acylating agent.

BACKGROUND ART

Today, semisynthetic  $\beta$ -lactam derivatives such as ampicillin, amoxicillin, cefaclor, cephalexin, cephadroxil and cephaloglycin are, in an industrial scale, prepared by chemical methods, for example by reacting an amino  $\beta$ -lactam such as 6-aminopenicillanic acid, usually having its carboxyl group protected, with an activated side chain derivative, followed by the removal of the protecting group by hydrolysis. For example, ampicillin (6-D- $\alpha$ -aminophenylacetamido-penicillanic acid) can be prepared by reacting 6-APA, having a suitable protected carboxyl group, with D-phenylglycine acid chloride, followed by removal of the protecting group by hydrolysis. These reactions typically involve costly steps such as sub zero degree Celcius conditions and organic solvents like methylene chloride and silylation reagents.

20 Within the last years, there has been an increasing amount of publications concerning the possibility of enzymatic preparation of penicillins and cephalosporins by reaction of an acylating agent, be it in acid form or in activated form (for example, amide or ester) and the parent amino  $\beta$ -lactam (for example, 6-APA or 7-ADCA). For example, enzymatic production of ampicillin from 6-APA and a D-phenylglycine derivative (such as a lower alkyl ester) is known

from West German patent application having publication No. 2,163,792, Austrian Patent No. 243,986, Dutch patent application No. 70-09138, West German patent application having publication No. 2,621,618, European patent application having publication No. 339,751 and PCT patent application having publication No. 92/01061.

10 The enzymatic acylation of an amino  $\beta$ -lactam with an acylating agent may be performed in the presence of a suitable amidase or acylase whereby the desired  $\beta$ -lactam derivative is formed. The parent amino  $\beta$ -lactam and the  $\beta$ -lactam derivative have the same  $\beta$ -lactam nucleus. In the  $\beta$ -lactam derivative endproduct the 6-NH<sub>2</sub> side chain of the penem parent  $\beta$ -lactam or the 7-NH<sub>2</sub> side chain of the cephem parent  $\beta$ -lactam are acylated.

15 This reaction can be illustrated by the following reaction scheme I:

Reaction scheme I

Acylation agent + amino  $\beta$ -lactam -  $\beta$ -lactam derivative

20 If this process is performed batchwise, it has been discovered that at the beginning of the reaction, an increasing amount of  $\beta$ -lactam derivative is formed whereas, after a certain period of time, the amount of  $\beta$ -lactam derivative present in the reaction mixture is decreasing. The decomposition of the  $\beta$ -lactam derivative formed may be due to 25 hydrolysis thereof whereby amino  $\beta$ -lactam and the acid form of the acylating agent is formed. In addition, the enzyme present may decompose the starting acylating agent. Consequently, at least two sorts of decomposition may take place during such a reaction.

30 One object of this invention is to furnish a process whereby the molar ratio between the amount of  $\beta$ -lactam derivative

which can be recovered from the reaction mixture and the acid form of the acylating agent formed during the reaction is increased, compared with this ratio for the known processes.

#### BRIEF STATEMENT OF THIS INVENTION

5 It has now, surprisingly, been found that improved process conditions are obtained if the enzymatic acylation of the amino  $\beta$ -lactam is performed at constantly high concentrations of both the parent amino  $\beta$ -lactam and the corresponding acylating agent. Preferably the concentrations of the parent 10 amino  $\beta$ -lactam and of the corresponding acylating agent are higher than 70%, preferably higher than 85% of the lowest value of both the saturated concentration of the parent amino  $\beta$ -lactam and of the saturated concentration of the corresponding acylating agent.

15 The advantages of this invention are, inter alia, as follows:

1) An improved molar ratio between the amount of  $\beta$ -lactam derivative which can be recovered from the reaction mixture and the amount of the acid form of the acylating agent, compared with this ratio for the known processes, is obtained. This improvement has direct impact on the economy 20 of the process due to the reduced consumption of acylating agent versus  $\beta$ -lactam derivative which can be recovered from the reaction mixture. This improvement also has an indirect impact on the economy of the process due to the lower amount 25 of the acid form of the acylating agent which is to be removed from the desired  $\beta$ -lactam derivative.

2) An increased yield of the  $\beta$ -lactam derivative is obtained.

30 3) An increase in the productivity of the  $\beta$ -lactam derivative is obtained. This productivity is defined as the

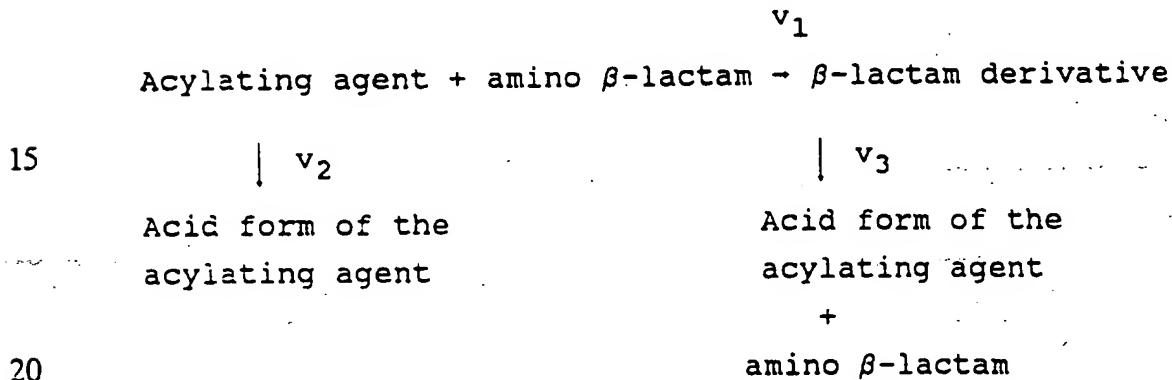
amount of  $\beta$ -lactam derivative which can be recovered per time unit per volume unit of the reaction mixture.

#### DETAILED DESCRIPTION OF THIS INVENTION

5       Herein, the terms (parent) amino  $\beta$ -lactam and acylating agent have been used for the two starting materials. The term  $\beta$ -lactam derivative has been used for the desired end product.

10       As mentioned above, two undesired decomposition reactions take place during the process in question and these reactions have been illustrated in the following reaction scheme II which also shows the parent reaction from reaction scheme I:

#### Reaction scheme II:



15       In reaction scheme II,  $v_1$  is the reaction rate for the reaction: acylating agent + amino  $\beta$ -lactam  $\rightarrow$   $\beta$ -lactam derivative,  $v_2$  is the reaction rate for the reaction: acylating agent  $\rightarrow$  acid form of the acylating agent, and  $v_3$  is the reaction rate for the reaction:  $\beta$ -lactam derivative  $\rightarrow$  acid form of the acylating agent + amino  $\beta$ -lactam.

20       One way of performing the process at constantly high concentrations of reactants according to this invention is to maintain the rate of formation of the desired  $\beta$ -lactam

derivative in the reaction mixture ( $v_1$ ) within a certain range for a certain period of time. Preferably, the rate of formation of the desired  $\beta$ -lactam derivative does, during a period of not less than 5 hours, preferably not less than 10 hours, not deviate more than  $\pm 50\%$ , more preferred not more than  $\pm 20\%$ , most preferred not more than  $\pm 10\%$ , from the average rate of formation of the desired  $\beta$ -lactam derivative during the same period of time.

To take an example, if the average rate of formation of amoxicillin during a period of 20 hours is 2.6  $\mu$ moles/minute/ml reaction mixture, then the rate of formation of amoxicillin during this period of time should preferably be within the range from 1.3 to 3.9  $\mu$ moles/minute/ml.

Another way of performing the process at constantly high concentrations of reactants according to this invention is to maintain the net rate of formation of the desired  $\beta$ -lactam derivative in the reaction mixture ( $v_1$  minus  $v_3$ ) within a certain range for a certain period of time. Preferably, the net rate of formation of the desired  $\beta$ -lactam derivative does, during a period of not less than 5 hours, preferably not less than 10 hours, not deviate more than  $\pm 50\%$ , more preferred not more than  $\pm 20\%$ , most preferred not more than  $\pm 10\%$ , from the average net rate of formation of the desired  $\beta$ -lactam derivative during the same period of time.

A still further way of performing the process at constantly high concentrations of reactants according to this invention is to maintain the ratio between, on one hand, the net rate of formation of the desired  $\beta$ -lactam derivative in the reaction mixture ( $v_1 - v_3$ ) and, on the other hand, the total rate of formation of the acid form of the acylating agent in the reaction mixture ( $v_2 + v_3$ ) within a certain range for a certain period of time. Hence, this ratio is  $(v_1 - v_3)/(v_2 + v_3)$ . Preferably, the ratio between the net rate of formation of the desired  $\beta$ -lactam derivative and the rate of formation of the acid form of the acylating agent does, during a period

of not less than 5 hours, preferably not less than 10 hours, not deviate more than  $\pm$  50 %, more preferred not more than  $\pm$  20 %, most preferred not more than  $\pm$  10 %, from the average ratio between the net rate of formation of the desired  $\beta$ -lactam derivative and the rate of formation of the acid form of the acylating agent during the same period of time.

5 The  $\beta$ -lactam derivative formed may precipitate during the reaction and, also, the acid form of the acylating agent such as D-phenylglycine and D-p-hydroxyphenylglycine may 10 precipitate. Hence, in some cases the reaction mixture will be a slurry throughout the reaction.

15 During the process of this invention, it is preferred that the total content of the desired  $\beta$ -lactam derivative, both in dissolved and optionally in precipitated form, in the reaction mixture is currently kept below a certain concentration. One object of doing so is to try to prevent that the viscosity of the reaction mixture becomes too high. Consequently, during the performance of the process at 20 constantly high concentrations of reactants of this invention, it is preferred that the total content of the desired  $\beta$ -lactam derivative, both in dissolved and optionally in precipitated form, does not exceed 350 mM/liter reaction mixture and preferably does not exceed 250 mM/liter reaction mixture.

25 The process according to this invention may be performed in a reactor which is equipped with a device for stirring or mixing and with means for maintaining the reaction temperature within a certain range. Also, it is recommended that this reactor be connected with an equipment maintaining 30 the pH value within a certain range.

During the continuous performance of the process, the amino  $\beta$ -lactam and the acylating agent may be added continuously or semicontinuously so as to maintain the desired concentrations thereof in the reaction mixture. The

resulting  $\beta$ -lactam derivative and the acid form of the acylating agent may be removed continuously or semicontinuously from the reactor, for example, by removal, from the reactor, of a part of the reaction mixture from which the resulting  $\beta$ -lactam derivative and, possibly, the acid form of the acylating agent is removed, for example, by centrifugation or filtration. The mother liquor from this centrifugation or filtration may be returned to the reactor or may be used to dissolve the amino  $\beta$ -lactam or the acylating agent.

Herein, the semicontinuous performance of an action means that the action in question is performed several times within a certain period of time, for example, during an hour or during a day and night.

The (parent) amino  $\beta$ -lactam has a free amino group which is acylated by the reaction according to this invention. The amino  $\beta$ -lactam may conveniently be 6-APA, 7-ADCA, 7-ACA or 7-ACCC.

The amino  $\beta$ -lactam, for example 6-APA or 7-ADCA, used in the process of this invention may be obtained by enzymatic hydrolysis of the fermented penicillins or cephalosporins, (for example penicillin V, penicillin G or cephalosporin C) or their ring enlarged analogues (for example V-DCA and G-DCA) or derivatives thereof followed by removal of the hydrolysis by-product, if desired (phenoxyacetic acid etc.). Advantageously, the crude solution can be used directly without further purification or dilution.

The acylating agent may be in an activated form. Preferably, the acylating agent is an amide or an ester. The acylating agent may be a derivative of D-phenylglycine, D-p-hydroxyphenylglycine, D-2,5-dihydrophenylglycine or mandelic acid, such as a lower alkyl ester (methyl, ethyl, n-propyl or isopropyl ester) or an amide which is unsubstituted or substituted in the  $-\text{CONH}_2$  group. The acylating agent may be

used in the form of a salt, for example, the hydrochloride salt or the  $H_2SO_4$  salt. The acylating agent may be added in an active form or the active form may be formed in situ.

5 The solubility of the acylating agent such as the D-phenylglycine or D-p-hydroxyphenylglycine derivative will vary with the identity of the derivative and with the composition of the reaction medium. In an aqueous system as used in the examples, the solubility of the hydrochloride salt of D-phenylglycine amide is typically approximately 450 mM. However, the solubility is very dependent on the salt components in the solution, as well as on the pH value and the temperature of the solution. As a further example, the solubility of the sulphate form of D-phenylglycine amide is about 3.3 M within a pH range from 2.5 to 6.5.

10 15 As appears from the above explanation, the acylating agent and the  $\beta$ -lactam derivative may be decomposed forming, inter alia, the acid form of the acylating agent. The acid form of the acylating agent is the parent carboxylic acid corresponding to the acylating agent in question.

20 Examples of  $\beta$ -lactam derivatives that may be prepared by the process of this invention are ampicillin, amoxicillin, cefaclor, cephalexin, cephadroxil, cephradine, epicillin and cefamandol.

25 The enzyme to be used in the process of this invention may be any enzyme catalyzing the reaction in question. Such enzymes have been known since around 1961. Enzymes to be used are, for example, termed penicillin amidase or penicillin acylase and classified as E.C. 3.5.1.11. A number of microbial enzymes are known to have this activity, derived from for example Acetobacter, Alcaligenes, Xanthomonas, Mycobacteria, Protaminobacter, Aeromonas (West German patent application having publication No. 2,163,792) Pseudomonas (Austrian Patent No. 243986), Flavobacterium (Dutch patent application No. 70-09138), Aphanocladium, Cephalosporium (West German

5 patent application having publication No. 2,621,618), Acetobacter pasteurianum, Alcaligenes faecalis, Bacillus megaterium, Xanthomonas citrii (European patent application having publication No. 339,751), Kluyvera citrophila (Agr.Biol.Chem. 37 (1973), 2797 - 2804) and Escherichia coli (West German patent application having publication No. 2,930,794). The Escherichia coli enzyme is commercially available. The enzyme also may be a so-called ampicillin hydrolase, acylase or amidase. In this connection, reference 10 is, inter alia, made to Hakko to Kogyo 38 (1980), 216 et seq., the content of which is incorporated by reference.

15 It is preferred to use the enzyme in a reusable form, for example, in entrapped or immobilized form. Immobilization may be done by any known method. Immobilized Escherichia coli enzyme is commercially available from Boehringer Mannheim GmbH, Germany, under the trade name Enzygel®, and Recordati under the trade name Super Enzyme®. Preferably, as enzyme Penicillin G acylase, immobilized on a 20 carrier consisting of a gelling polycarbohydrate and a polymer containing free amino groups, is applied.

The process of this invention is generally carried out in a system containing water. If desired, an organic solvent may be added.

25 Generally, the reaction temperature of the process of this invention may vary between 0 and 35°C, especially between 10 and 30°C. Temperatures in the range from 20 to 30°C may be preferred for convenient operation.

30 The suitable pH value depends, inter alia, on the type of the enzyme used. Using Escherichia coli enzyme, the pH value is typically in the range from 5 to 8, preferably in the range from 6.1 to 7. For the preparation of amoxicillin, a pH value in the range from 5.5 to 6.4 is preferred. Control of the pH value may be used.

35 Suitable reaction times are above 5 hours. The reaction time may be days or weeks.

Suitable enzyme concentrations may be from 1 to 100 U/ml (1 U = one unit of enzyme activity, see below).

Before the process is started, the reactor may be filled with the components in the desired amounts.

Using the process according to this invention, an extraordinary high molar ratio between the amount of  $\beta$ -lactam derivative which can be recovered and the total amount of acid form of the acylating agent can be obtained. These high ratios are obtained using the teachings of this invention and properly selecting the concentration of the acylating agent, the ratio between the concentration of acylating agent and the starting amino  $\beta$ -lactam, the pH value and the enzyme. Thus, a ratio of 2.4 was obtained in Example 1 below using the process according to the present invention. In a comparative batch process, vide Example 1 below, a molar ratio of only 1.4 was obtained. In addition, the yields of isolated product obtained in this example were 90 and 85 %, respectively.

Purification of the product can be achieved by methods known per se, for example by crystallisation.

Herein, the following abbreviations have been used: 7-ACA is 7-aminocephalosporanic acid, 7-ACCC is 7-amino-3-chloro-3-cephem-4-carboxylate, 7-ADCA is 7-aminodesacetoxycephalosporanic acid, 6-APA is 6-aminopenicillanic acid, D-HPG is D-p-hydroxyphenylglycine, D-PG is D-phenylglycine, D-PGA is D-phenylglycine amide, D-PGM is D-phenylglycine methyl ester, D-HPGM is D-p-hydroxyphenylglycine methyl ester, G-DCA is 7-phenylacetamidodesacetoxycephalosporanic acid, D-HPGA is D-p-hydroxyphenylglycine amide, and V-DCA is 7-phenoxyacetamido-desacetoxycephalosporanic acid.

Any novel feature or combination of features described herein is material to this invention.

This invention is further illustrated by the following examples which, however, are not to be construed as limiting.

## DEFINITIONS and METHODS of ANALYSIS

5 Enzyme:

As enzyme Penicillin G acylase (26250 U, size 200-500  $\mu$ m) isolated according to WO application 012782 immobilized on a carrier consisting of a gelling carbohydrate and a polymer containing free amino groups according to European patent 22642, is applied.

As definition of penicillin G acylase activity the following is used: one unit (U) corresponds to the amount of enzyme that hydrolyses per minute 1  $\mu$ mole penicillin G under standard conditions (100 g.l<sup>-1</sup> Penicillin G potassium salt, 0.05 M potassium phosphate buffer, pH value 8.0, 28°C).

### HPLC analysis of ampicillin and cephalexin:

Column: RP LC-18, (250 x 4.6 mm; 5 $\mu$ m)

Eluent A: 25 mM phosphate buffer, pH value 6.5

Eluent B: acetonitrile

20 Gradient: Time, minutes eluent B, %

Flow: 1 ml/min. Detection: 215 nm.

25 Retention times in minutes: 4.1 (D-PG); 6.3 (7-ADCA); 8.1 (6-  
APA); 9.1 (D-PGA); 13.4 (Cephalexin); 13.9 (Ampicillin); 18  
(D-PGM).

HPLC analysis of amoxicillin:

Column: Chromspher C18, 5  $\mu$ m (100 x 3.0 mm)

Solvent: 25 % acetonitrile in 12 mM phosphate buffer + 0.2% sodiumdodecylsulphate, pH value: 2.6. Flow: 1 ml/min. UV-detection at 214 nm.

Retention times in minutes: 1.9 (D- $\alpha$ -hydroxyphenylglycine); 7.3 (D-HPGM); 3.1 (D-HPGA); 3.4 (6-APA); 4.8 (Amoxicillin).

## EXAMPLE 1

## ENZYMATIC PREPARATION OF AMOXICILLIN FROM D-HPGM AND 6-APA

10 The equipment for this experiment consisted of (see Fig. 1) a thermostated reactor having a volume of 1.5 liters, equipped with a three-bladed impeller and a sieve with slots 180  $\mu$ m (open area about 32%). The reactor was connected to an autotitrator system using 4 M sulphuric acid as titrand. A 15 valve was positioned at the outlet of the reactor. The outlet of the valve was connected via a pump to a basket centrifuge equipped with a polypropylene bag having a density of 1-5  $\mu$ m. The outlet from the centrifuge was connected via a pump to a feed tank equipped with a stirrer and a glass sinter bottom. 20 The outlet from the feed tank was connected via a pump to the reactor.

25 A mixture consisting D-HPGM (36.2 g, 200 mmol) and 6-APA (43.2 g, 200 mmol) in 800 ml water was added to the reactor with the bottom valve closed. The stirring was started. Immobilized Penicillin G acylase (26250 U, size 200-500  $\mu$ m) made up to 200 ml was added to the reactor. The pH value was maintained at 6.1. The reaction temperature was about 20 °C. Under these conditions, the reaction mixture was almost saturated with D-HPGM and 6-APA. Then, the bottom valve was 30 opened allowing the reaction mixture from the reactor to enter the centrifuge. Thereafter, the mother liquor from the centrifuge was pumped into the feed tank wherein D-HPGM

5 (21.7 g, 120 mmol) and 6-APA (20.0 g, 92.5 mmol) were loaded. The total volume of the suspension in the feed tank was kept at about 75 ml. A flow of about 100 ml/min was maintained in the system. The concentration of reaction components in the reactor, in the reactor outlet, in the centrifuge outlet, in the feed tank and in the feed tank outlet were monitored by analytical HPLC.

10 As the reaction proceeded, the amoxicillin and D-HPG formed started to precipitate out of solution. The crystals were separated from the immobilized enzyme particles by the bottom sieve in the reactor and the crystal suspension was led to the centrifuge where the crystals were separated from the mother liquor. When the mother liquor, which was now undersaturated with respect to D-HPGM and 6-APA, passed 15 through the feed tank, some of the solid D-HPGA and 6-APA present dissolved such that the outlet from the feed tank contained saturated D-HPGM and 6-APA. When the total concentrations of D-HPGM and 6-APA came down to about 225 mM HPGM and 225 mM 6-APA, more solid substrate was added to the feed tank. At intervals the crystals in the centrifuge were 20 washed with water, the washing liquid was added to the reactor. The amount of water used was sufficient to keep the volume in the reactor at its starting level. After washing the crystals in the centrifuge, the centrifuge was emptied.

25 After about 12 hours, the dosing of D-HPGM and 6-APA was stopped. After 14 hours, the 6-APA concentration reached 20 mM and the reaction was stopped. The amounts of reaction components are given in Table 1, below.

Table 1.

	substrate added	crystal cake at the end	in solution at the end
D-HPGM	606.5 g (3351 mmol)	2.0 g (11 mmol)	10.9 g (60 mmol)
6-APA	523.4 g (2423 mmol)	9.0 g (42 mmol)	4.3 g (20 mmol)
Amoxicillin. 3H <sub>2</sub> O	0	909.6 g (2171 mmol)	8.4 g (20 mmol)
D-HPG	0	123.7 g (741 mmol)	30.0 g (180 mmol)

Based on the above results, the following yield can be calculated: amoxicillin yield (isolated product, based on 6-APA added): 90%.

10 Amoxicillin productivity: 156 mmol/hour/liter.

Hereinafter, the molar ratio between the amount of  $\beta$ -latam derivative which is formed and the amount of the acid form of the acylating agent formed has been designated R. In this example R = 2.4.

15 A comparative experiment was performed at batchwise conditions and the reaction was stopped at the moment where the optimum yield of amoxicillin was obtained (2 hours). The reaction temperature was about 20 °C, the pH value was about 6.1 and 26250 units of the enzyme, mentioned in this example above, was used. The total volume of the reaction mixture was 20 1 liter. The reaction was performed in the reactor mentioned above. After 2 hours the bottom valve was opened, and the crystals were separated from the immobilized enzyme particles by the bottom sieve in the reactor. The crystal suspension

was filtered. The amounts of reaction components are given in Table 2, below.

Table 2.

	substrate added	crystal cake at the end	in solution at the end
D-HPGM	90.5 g (500 mmol)	0.1 g (0.6 mmol)	34.2 g (189 mmol)
6-APA	43.2 g (200 mmol)	0.7 g (3 mmol)	3.0 g (14 mmol)
Amoxicillin. 3H <sub>2</sub> O	0	70.8 g (169 mmol)	4.2 g (10 mmol)
D-HPG	0	0.3 g (2 mmol)	21.7 g (130 mmol)

Amoxicillin yield (isolated product, based on 6-APA added):  
10 85%.

Amoxicillin productivity: 90 mmol/hour/liter.

R = 1.4.

#### EXAMPLE 2

##### ENZYMATIC PREPARATION OF AMOXYCILLIN FROM D-HPGA AND 6-APA

15 The equipment for this experiment is described in example 1.

A mixture consisting of D-HPGA (20.0 g, 120 mmol) and 6-APA in 800 ml water, which was adjusted at a pH value of 6.0 by adding 4 M ammonium hydroxide, was added to the reactor with the bottom valve closed. The stirring was started.  
20 Immobilized Penicillin G acylase (26250 U, size 200-500  $\mu$ m) made up to 200 ml was added to the reactor. The pH value was

5 maintained at 6.0. The reaction temperature was about 20 °C. Under these conditions the reaction mixture was almost saturated with D-HPGA and 6-APA. Then the bottom valve was opened allowing the reaction mixture from the reactor to enter the centrifuge. Thereafter, the mother liquor from the centrifuge was pumped into the feed tank wherein D-HPGA (20.0 g, 120 mmol) and 6-APA (20.0 g, 92.5 mmol) were loaded. The total volume of the suspension in the feed tank was kept at about 75 ml. A flow of about 100 ml/min was maintained in the system. The concentration of reaction components in the reactor, in the reactor outlet, in the centrifuge outlet, in the feed tank, and in the feed tank outlet were monitored by analytical HPLC.

10 15 The crystals formed in the reactor were separated from the immobilized enzyme particles by the bottom sieve in the reactor. The crystal suspension was led to the centrifuge where the crystals were separated from the mother liquor. The mother liquor was passed through the feed tank. When the total concentrations in the feed tank came down to about 150 mM HPGA and 225 mM 6-APA, more solid was added to the feed tank. At intervals the crystals in the centrifuge were washed 20 with water, the washing liquid was added to the reactor. The amount of water used was sufficient to keep the volume in the reactor at its starting level. After washing the crystals in 25 the centrifuge, the centrifuge was emptied.

After about 12 hours, the dosing of 6-APA to the feed tank was stopped while the D-HPGA dosing was maintained in order to keep D-HPGA at saturation. After 14 hours, the 6-APA concentration reached 20 mM and the reaction was stopped. The 30 amounts of reaction components are given in Table 3, below.

Table 3.

	substrate added	crystal at the end	in solution at the end
D-HPGA	483.9 g (2915 mmol)	1.3 g (8 mmol)	22.4 g (135 mmol)
6-APA	443.0 g (2051 mmol)	8.0 g (37 mmol)	4.3 g (20 mmol)
Amoxicillin. 3H <sub>2</sub> O	0	758.4 g (1810 mmol)	8.4 g (20 mmol)
D-HPG	0	103.2 g (618 mmol)	29.7 g (178 mmol)

5

Amoxicillin yield (isolated product, based on 6-APA added):  
88%.

Amoxicillin productivity: 131 mmol/hour/liter.

10

R = 2.3.

## CLAIMS

1. A process for preparing a  $\beta$ -lactam derivative by enzymatic reaction of the parent amino  $\beta$ -lactam with the corresponding acylating agent, characterized in that the reaction is performed at constantly high concentrations of both the parent amino  $\beta$ -lactam and the corresponding acylating agent.

2. A process according to claim 1 wherein both the amino  $\beta$ -lactam and the acylating agent are added either continuously or semicontinuously.

3. A process according to claim 1, wherein the ratio between, on one hand, the net rate of formation of the desired  $\beta$ -lactam derivative in the reaction mixture ( $v_1 - v_3$ ) and, on the other hand, the total rate of formation of the acid form of the acylating agent in the reaction mixture ( $v_2 + v_3$ ), for a period of about 5 hours, preferably of about 10 hours, does not deviate more than about  $\pm 50\%$ , more preferred not more than about  $\pm 20\%$ , most preferred not more than about  $\pm 10\%$ , from the average ratio between the net rate of formation of the desired  $\beta$ -lactam derivative and the total rate of formation of the acid form of the acylating agent during the same period of time.

4. A process according to claim 1, wherein the rate of formation of the desired  $\beta$ -lactam derivative in the reaction mixture ( $v_1$ ), for a period of about 5 hours, preferably about 10 hours, does not deviate more than about  $\pm 50\%$ , more preferred not more than about  $\pm 20\%$ , most preferred not more than about  $\pm 10\%$ , from the average rate of formation of the desired  $\beta$ -lactam derivative during the same period of time.

5. A process according to any one of the preceding claims, wherein the  $\beta$ -lactam derivative and optionally the acid form of the acylating agent is continuously or semicontinuously removed during the reaction.

5 6. A process according to any one of the preceding claims, wherein the total content of dissolved and optionally precipitated  $\beta$ -lactam derivative does not exceed the amount of 350 mMol/liter reaction mixture.

10 7. A process according to any one of the preceding claims, wherein the parent amino  $\beta$ -lactam is selected from the group consisting of 6-aminopenicillanic acid, 7-aminodes-acetoxycephalosporanic acid, 7-aminocephalosporanic acid, 7-amino-3-chloro-3-cephem-4-carboxylate, and 7-amino-3-[[[(1-methyl-1H-tetrazol-5-yl)thio]methyl]-3-cephem-4-carboxylate.

15 8. A process according to any one of the claims 1-7, wherein the acylating agent is selected from the group consisting of a derivative of D-phenylglycine, a derivative of D-p-hydroxyphenylglycine, a derivative of D-2,5-dihydrophenylglycine or a derivative of mandelic acid.

20 9. A process according to any one of the claims 1-6, wherein the resulting  $\beta$ -lactam derivative is selected from the group consisting of ampicillin, amoxicillin, cefaclor, cephalexin, cephadroxil, cephadrine, epicillin, and cefamandol.

25 10. A process according to any one of the preceding claims, wherein the reaction is performed at a temperature in the range from about 0 to about 35°C, preferably above about 10°C.

30 11. A process according to any one of the preceding claims, wherein the reaction is performed at a pH value in the range from above about 5 through about 8.

12. A process according to any one of the preceding claims, wherein the starting amino  $\beta$ -lactam is prepared by hydrolysis of penicillin V, penicillin G, 7-phenoxyacetamido-desacetoxycephalosporanic acid, 7-phenylacetamidodesacetoxycephalosporanic acid or Cephalosporin C or a derivative thereof and, optionally, removal of the side chain liberated by the hydrolysis.

13. A process according to any one of the preceding claims, wherein the acylating agent is an amide, preferably an amide wherein the  $-\text{CONH}_2$  group is unsubstituted, or an ester containing 1 - 3 carbon atoms in the ester part.

14. A process according to any one of the preceding claims, wherein the enzyme used is from *Escherichia coli*, *Acetobacter pasteurianum*, *Xanthomonas citrii*, *Kluyvera cirophila*, *Bacillus megaterium* or *Alcaligenes faecalis*.

15. A process according to any one of the preceding claims, wherein an enzymatically active reusable material is used.

16. A process according to any one of the preceding claims, wherein the enzyme used is immobilized.

17. A process according to any one of the preceding claims, wherein the enzymatic reaction is carried out in an aqueous system, optionally together with an organic solvent.

1 / 1

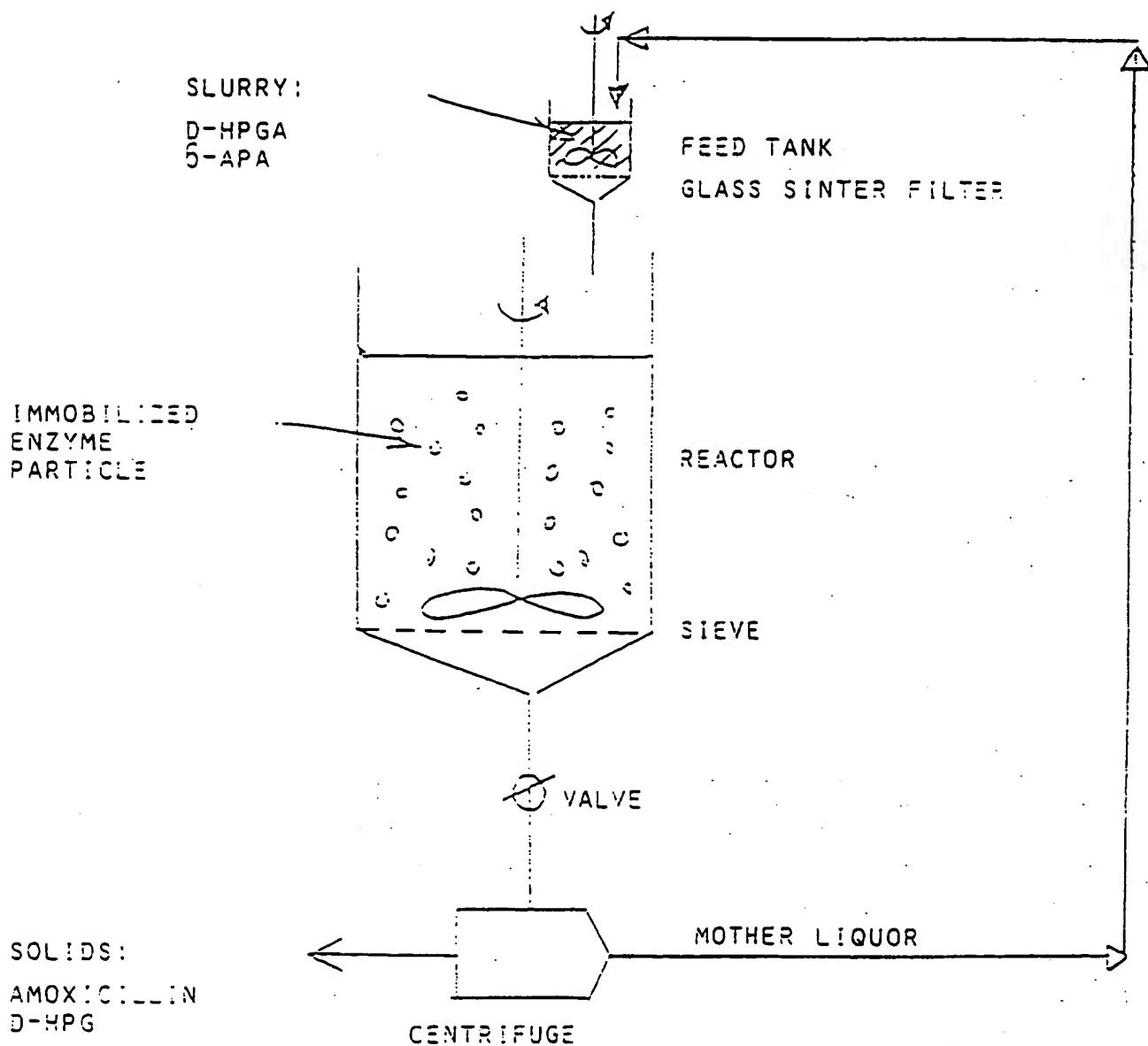


FIGURE 1

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 95/02876

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12P37/04 C12P35/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 01061 (NOVO NORDISK A/S) 23 January 1992 cited in the application see the whole document ---	1-17
Y	WO,A,91 09136 (CONSEJO SUPERIOR INVESTIGACIONES CIENTIFICAS) 27 June 1991 see the whole document ---	1-17
A	EP,A,0 339 751 (GIST-BROCADES N. V.) 2 November 1989 cited in the application see the whole document -----	1-17

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

17 October 1995

Date of mailing of the international search report

13.11.95

Name and mailing address of the ISA

European Patent Office, P.O. 5818 Patentlaan 2  
 NL - 2280 IJ1V Rijswijk  
 Tel. (+ 31-70) 340-2040, Tx. 31 651 cpo nl,  
 Fax (+ 31-70) 340-3016

Authorized officer

Chouly, J.

**INTERNATIONAL SEARCH REPORT**

Int'l Application No

**PCT/EP 95/02876**

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9201061	23-01-92	AU-B- CA-A- EP-A-	8208691 2086250 0537255	04-02-92 05-01-92 21-04-93
WO-A-9109136	27-06-91	AU-B- US-A-	6960291 5268271	18-07-91 07-12-93
EP-A-339751	02-11-89	JP-A- PT-B-	1312998 90373	18-12-89 30-09-94

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**